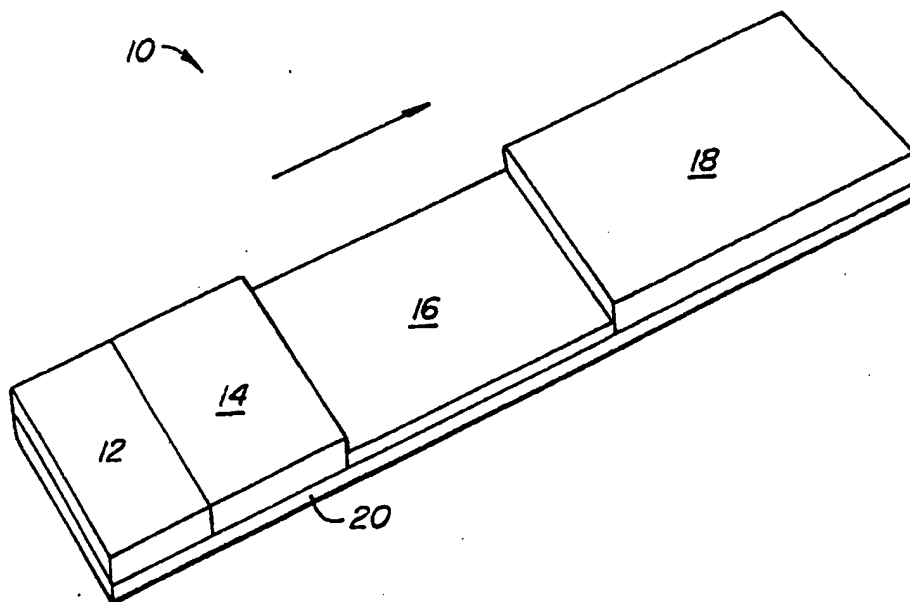




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(54) Title: IMMUNOASSAY USING DYE COMPLEXED ENZYME CONJUGATES



(57) Abstract

Target-specific dye labeled complexes are formed by reacting a conjugate of an enzyme and a specific binding substance, such as an antibody or antigen, with an enzyme substrate in a liquid medium under conditions where the substrate is converted to dye molecules which then complex to the conjugate. Such dye labeled complexes are useful in a variety of biological assays, particularly lateral flow assays where the dye labeled complex is immobilized within a label zone (14) which forms part of a flow path for a liquid sample or liquid reagent in a test device (10). Liquid flow through the label zone (14) transports the dye labeled complex to a subsequent capture zone (16) where it is bound in proportion to the presence or absence of a target analyte within the liquid sample.

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IMMUNOASSAY USING DYE COMPLEXED ENZYME CONJUGATES

5

BACKGROUND OF THE INVENTION1. Field of the Invention

10 The present invention relates generally to compositions and methods for their preparation and use in biological assay systems. More particularly, the present invention relates to target-specific labeling complexes comprising a specific binding substance, an enzyme, and dye molecules complexed thereto.

15 The use of chromogenic and fluorescent dyes as "labels" in biological assay procedures is known. Typical assay protocols call for direct or indirect binding of a dye label to an analyte or analyte analog in a biological sample, where the presence or absence of the dye at a particular stage
20 of the assay can be determined visually and related to the amount of analyte initially present in the sample. A wide variety of specific assay protocols exist.

Of particular interest to the present invention, certain assays utilize naturally colored or dyed particles as a
25 label, where the particles are bound to an antibody or other specific binding substance. Suggested particles include dyed latex beads, dyed liposomes, erythrocytes, metal sols, and the like. The colored particle in such complexes can serve as a visible marker, where separation, capture, or aggregation of
30 the particles is mediated through binding of the antibody or other specific binding substance. The amount of label thus segregated in a particular assay step is related to the amount of analyte initially present in the sample.

A variety of methods for preparing such antibody-
35 particle compositions have been proposed. Such methods generally rely on producing a colored particle, typically by dyeing a latex bead, a liposome, or the like, and subsequently

attaching the colored particle to the antibody, typically by passive adsorption or by covalent binding.

While generally useful, methods for preparing such antibody-particle compositions can be relatively complex, usually requiring multi-stage operations including preparation of the particle, coloring of the particle, attachment of the particle to the antibody, and blocking of the particle for use in immunoassays. Moreover, a loss of antibody binding capacity can often result from the particle attachment. Sometimes these particles are not compatible with the antibodies selected for a particular application.

It would be desirable to provide improved labeling compositions and methods for their production that would allow for any antibody to be used for an application. The compositions should be relatively easy to prepare, with a reduced cost, and have uniform characteristics. In particular the compositions should retain antibody activity to a significant extent. It will be appreciated, however, that the methods and compositions of the present invention need not be superior to the prior art in each or any of these aspects, but rather that these are general advantages that the present invention can provide relative to certain prior art methods and products.

2. Description of the Background Art

U.S. Patent No. 4,863,875, describes compositions comprising at least ten dye molecules or monomers covalently attached to an antibody through an isocyanate group on the dye. U.S. Patent No. 4,452,886, describes the covalent attachment of photon absorbing or emitting polymers to proteins, such as antibodies and antigens. U.S. Patent No. 4,373,932, describes labeling of a ligand with an aqueous dispersion of a hydrophobic dye or pigment, or a polymer nuclei coated with such a dye or pigment. U.S. Patent No. 4,703,017, describes a solid phase assay device which relies on specific binding of a ligand-label conjugate on a solid support, where the label is disclosed as a particulate, such as a sac, erythrocyte, erythrocyte ghost, liposome, or polymer microcapsule. U.S. Patent No. 4,943,522, describes a solid phase lateral flow

assay using erythrocytes as a label. U.S. Patent No. 4,861,711, describes a solid phase lateral flow assay using enzyme antibody conjugate and substrate, each separately held in absorbant pads. See also, U.K. Patent No. 2,204,398; EP
5 Patent No. 306 722; and EP Patent No. 276 152, which relate to lateral flow assays. Enzyme assays and immunohistochemical staining techniques which produce a colored dye by reaction of a substrate with an enzyme bound to a target moiety are known.

SUMMARY OF THE INVENTION

10 The present invention provides improved target-specific labeling complexes for use in biological assays, such as lateral flow assays as described hereinafter. The target-specific complexes comprise an enzyme component bound to a
15 specific binding substance component, such as an antibody or antigen, and dye molecules complexed to the enzyme and/or specific binding substance in an amount sufficient to impart a visually discernible color to the complex. The complexes may result from either covalent or non-covalent attachment of the dye to the enzyme component, or both. The complexed dye
20 molecules do not significantly block active site(s) on the specific binding substance which thus remain available for binding to an analyte or other target molecule in the biological assays. Usually, the enzyme will be inactivated so that native enzyme activity is removed prior to use as a
25 labeling complex according to the present invention. Advantageously, the complexed dye molecules are lyophilized and remain intact, even when introduced to an aqueous reaction medium, as described hereinafter.

The number of complexed dye molecules or moieties may
30 vary wide, from as few as 10 per complex to as many as 10^7 per complex, with the primary requirement being that there be sufficient dye to render the particle visible in the biological assay in which it is employed. Usually, each complex will comprise from 10^2 to 10^4 dye molecules or moieties, with the
35 individual molecule weight of each moiety being between 100 and 1000 daltons (although the weight may be as high as 10^6 daltons). The aggregate size of the complex will usually lie

between 10^3 and 10^{11} daltons, more usually being between 10^6 and 10^{10} daltons.

According to a method of the present invention, the target-specific labeling complexes are formed in a aqueous medium by reacting the enzyme and specific binding substance components with an enzyme substrate, where the enzyme converts the substrate to a dye. The dye thus formed in the aqueous medium complexes to the conjugate, and the enzyme reaction is stopped at a time when the color of the labeling complex is sufficiently intense to be readily observable when used in assays as described hereinafter. Conveniently, the reaction may be stopped either by substrate exhaustion and/or by altering the reaction conditions. The method may further comprise inactivating the enzyme and/or lyophilizing the complex after the desired color intensity has been imparted.

In another aspect of the present invention, test articles comprise the target-specific labeling complex impregnated, usually by lyophilization, in a support matrix, usually a nonbibulous or bibulous solid matrix capable of conducting liquid flow, preferably in a single direction in the matrix. In this way, a liquid sample can be applied to the solid matrix and will flow through the matrix so that impregnated labeling complex can be transported by the liquid flow to a "downstream" location. Advantageously, the labeling complexes of the present invention have been found to remain intact when transported by liquid flow as just described.

Typically, an assay can be run using the test article based on capture of the labeling complex mediated by the specific binding substance. Usually, a region of the test article will incorporate an immobilized capture reagent capable of directly or indirectly binding the labeling complex. The assay result will then depend on appearance of the label within this capture zone.

Alternatively, the labeling complex may be initially bound to the capture reagent within the "capture zone" by an antibody or other binding substance specific for an analyte. By then applying a sample to the support matrix, flow of the sample through the capture zone will displace the labeling

complex as a result of competitive binding with analyte present in the sample. Thus, the color initially present in the capture zone will disappear in proportion to the amount of analyte present in the sample.

5 In yet another aspect of the present invention, an assay device for detecting the presence of analyte in a liquid sample comprises a sample receiving zone, optionally a labeling zone, a capture zone, and an absorbent zone. The sample is applied to the receiving zone and flows through the device
10 where it combines with the labeling complex within the labeling zone or displaces the labeling complex from the capture zone. In the first case, the labeling complex either binds to or competes with analyte in the sample, and the label is bound within the capture zone depending on the amount of analyte
15 originally present in the sample. In the second case, no labeling zone is necessary and the analyte in the sample displaces the labeling complex into the absorbent zone. In both cases, the absorbent zone acts to receive liquid sample as it flows through the previous zones within the device.

20 DESCRIPTION OF THE DRAWINGS

Fig. 1 illustrates an assay device incorporating the principles of the present invention, specific examples of which are described in detail in the Experimental section hereinafter.

25 DESCRIPTION OF SPECIFIC EMBODIMENTS

The present invention provides compositions, methods for preparing such compositions, test articles, and test devices for use in performing biological assays, particularly immunoassays. The compositions comprise target-specific
30 labeling complexes which are visually discernible, typically being colored and capable of providing a readily apparent color signal when aggregated or accumulated in a capture zone, as described in more detail hereinafter. The compositions further comprise a specific binding substance which mediates such
35 capture within the capture zone, and the compositions can be utilized in a wide variety of particular assay formats and protocols. While the compositions are particularly valuable

for use in lateral flow assay protocols, they will find use in a wide variety of other assay formats as well.

The compositions of the present invention are composed of a conjugate including an enzyme component and a specific binding substance component, where the enzyme component is selected to provide for conversion of appropriate substrate(s) into a dye substance which will complex to the conjugate as it is produced in an aqueous medium. Suitable dyes should have a high avidity for the complex, with covalently binding dyes being preferred. Dyes which do not bind covalently should interact with the complex through hydrophobic, ionic, and other non-covalent mechanisms which result in strong binding, particularly to the protein components of the complex. The selection of a particular enzyme and substrate system to produce a particular dye will depend on the particular application, desired color, and the like.

Exemplary enzyme-substrate systems are set forth in Table A.

20	<u>ENZYME</u>	<u>TABLE A</u> <u>SUBSTRATE(S)</u>	<u>COLORED PRODUCT</u>
	OXIDASES:		
	Glucose Oxidase	phenazine methosulfate tetrazolium salt	formazan
25	PEROXIDASES:		
	Horse radish peroxidase	aminoethylcarbazole; hydrogen peroxide	red
30	Horseradish peroxidase	benzidine; hydrogen peroxide	brown
	Horseradish peroxidase	chloronaphthol; hydrogen peroxide	blue/gray
35	Horseradish peroxidase	chloronaphtol; hydrogen peroxide; MBTH	purple indamine dye
40	Horseradish peroxidase	chlornaphthol; hydrogen peroxide; 4-aminoantipyrine	red quinoneimine dye
45	PHOSPHATASES: Alkaline phosphatase	indolyl phosphate	indigo

	Alkaline phosphatase	azonaphthol phosphate	azonaphthol
5	Alkaline phosphatase	indolyl phosphate; tetrazolium	formazan-indigo complex
	Alkaline phosphatase	naphthol phosphate; diazo compound	azo compound
10	DIAPHORASE	tetrazolium; NADH	formazan
15	GALACTOSIDASES	indolyl galactoside azonaphthol galactoside	indigo azonaphthol
		indolyl galactoside; tetrazolium	formazan
20		naphthol galactoside; diazo compound	azo compound
25	OXIDOREDUCTASE	tetrazolium	formazan

The specific binding substance will be a compound having spatial and/or polar features which permit it to bind specifically to another compound. Specific binding substances useful in the present invention will be selected or prepared to specifically bind to particular compositions, such as analytes, anti-analyte substances, and other target substances where binding may be advantageously performed as part of a biological assay procedure. Natural specific binding pairs include antigens and antibodies, lectins and carbohydrates, hormones and hormone receptors, enzymes and enzyme substrates, biotin and avidin, vitamins and vitamin binding proteins, complementary polynucleotide sequences, drugs and receptors, enzymes and inhibitors, apoproteins and cofactors, growth factors and receptors, and the like. Biotin and avidin derivatives may also be used, including biotin analogs/avidin, biotin/streptavidin, and biotin analogs/streptavidin. When no natural specific binding substance exists, one may be prepared. For antigenic and haptenic target substances, antibodies may be prepared by well known techniques. For polynucleotides,

complementary DNA or RNA fragments may also be prepared by well known synthesis techniques.

The preferred specific binding substances are antibodies, antigen, and haptens, and the remaining discussion will be directed primarily at those substances. It will be appreciated, however, that the preparatory methods can easily be adapted to provide compositions employing other specific binding substances as described above. Additionally, the test articles and test devices of the present invention can easily be modified to employ target-specific complexes having specific binding substances other than antibodies and antigens, and the assay methods of the present invention can also be so modified.

The enzyme will be an oxidase, peroxidase, phosphatase, diaphorase, galactosidase, lytic enzyme, or oxidoreductase, with exemplary enzymes set forth in Table A, above. The enzyme will usually be covalently attached to the specific binding substance, but indirect linkage such as through a biotin-avidin binding or other cognate members of specific binding pairs may also find use. When the specific binding substance is a polypeptide or protein, such as an antibody, the enzyme may be covalently bound through a variety of moieties, including disulfide, hydroxyphenyl, amino, carboxyl, indole, or other functional groups, employing conventional conjugation chemistry as described in the scientific and patent literature. Binding should be effected in such a way that active site(s) on the specific binding substance are not blocked and remain available for binding to the desired target substance. In the case of antibodies, binding will preferably be effected so that the complementarity determining regions remain available for binding to the desired substance. Specific techniques for derivatizing antibodies for binding to enzymes are described in Tijssen, "Practice and Theory of Enzyme Immunoassays" in *Laboratory Techniques in Biochemistry and Molecular Biology*, vol. 15, Burdon and van Knippenberg, eds. 1985, Elsevier, Amsterdam, the disclosure of which is incorporated herein by reference.

Once the specific binding substance-enzyme conjugates are formed, they will be reacted with the appropriate enzyme

substrate(s) (see Table A) in aqueous medium under conditions sufficient to convert the substrate to a colored dye product. The reaction conditions are further selected so that the dye molecules produced by the enzyme conversion will form a complex
5 with the conjugate, with sufficient dye being incorporated into the complex to impart a visually discernible color.

Reaction conditions necessary to effect complexation depend on the nature of the enzyme and substrate that are chosen for a particular application. Each enzyme has reaction
10 conditions that are peculiar to it for optimal turnover and generation of dye product. Ideal candidate enzymes, chromogenic substrates, and reaction conditions (such as pH, ionic strength, temperature cofactors, and the like) are those that are employed for enzyme-antibody mediated histochemical
15 staining, described in chapter 17 of "Practice and Theory of Enzyme Immunoassays," *supra*.

The enzyme conversion and complexation reaction will be carried out until sufficient dye has been produced and complexed to the conjugate so that the resulting complex will
20 have a sufficiently intense color to be visually detectable when aggregated or bound within a capture zone, as described hereinafter. The reaction and complexation can be terminated by a variety of approaches. Conveniently, the amount of substrate reacted can be limited so that the substrate will be
25 exhausted when the complex color intensity is sufficient. Alternatively, the enzyme conversion of substrate to dye product can be quenched by altering the reaction conditions or adding an enzyme inhibitor at a time selected to terminate dye production. As a second alternative, it would be possible to
30 simply remove or separate particles from the substrate in the reaction medium in order to stop the reaction and/or remove soluble dye, for example, by using gel permeation chromatography.

Usually, but not necessarily, the enzyme activity
35 will be inhibited after complexation is completed, either by exposure to an enzyme inhibitor, exposure to denaturing conditions (where the conditions selected must not denature or inactivate the specific binding substance) exhaustion of an

enzyme cofactor, or the like. It will be appreciated that the ability of the target-specific labeling complex of the present invention derives from the color provided by the complexed dye molecules, not from the ability of the enzyme to turnover
5 substrate.

Unreacted substrate will preferably be removed from the labeling complexes after the reaction has been stopped, typically by chromatographic separation techniques, such as gel permeation. If the enzyme is not inactivated, it is essential
10 that substantially all substrate be separated in order to prevent additional dye production in the subsequent assay procedure. Usually, however, the enzyme will be inactivated in order to further avoid the possibility of unintended dye production.

15 In a preferred technique, the target-specific labeling complexes of the present invention will be applied to a solid phase matrix, more preferably being lyophilized within the matrix. The solid phase matrix may be bibulous or nonbibulous (as described in copending application Serial No.
20 07/639,967, the disclosure of which has previously been incorporated herein by reference) and will generally be able to conduct liquid flow therethrough so that the liquid may interact with the impregnated labeling complex.

Such matrices may be composed of a variety of
25 materials of the type generally employed for preparing porous membranes, absorptive pads, and the like, in the immunoassay art. Particularly preferred are fabrics composed of polyester, acrylonitrile copolymer, rayon, glass fiber, cellulose, and blends thereof. In certain embodiments it will be desirable to
30 treat such fabrics with a blocking agent to render the fabric nonbibulous, as generally described in copending application Serial No. 07/639,967, the disclosure of which has previously been incorporated herein by reference.

As an alternative to impregnation in a solid phase
35 matrix, the labeling complex particles of the present invention may be dried and lyophilized into a powder form. Such lyophilized labeling complexes are then available for use in assay protocols as generally known in the art.

Referring now to Fig. 1, an exemplary test device 10 constructed in accordance with the principles of the present invention will be described. Test device 10 will employ nonbibulous sample and reagent flow regions generally as described in copending application Serial No. 07/639,967, the disclosure of which has previously been incorporated herein by reference. It will be appreciated, however, that other test devices employing bibulous sample and reagent flow regions, or combinations of nonbibulous and bibulous regions, may be constructed for use with the target-specific labeling complexes of the present invention.

The test device 10 includes a sample receiving zone 12, a label zone 14, a capture zone 16, and an absorbent zone 18. The sample receiving zone 12, label zone 14, and capture zone 16 will be composed of materials capable of receiving liquid samples and other liquid reagents and transporting such samples and reagents in a lateral direction, i.e. from the receiving zone 12 toward the absorbent zone 18. The absorbent zone 18 will be composed of a material capable of receiving and absorbing the same liquid samples and reagents. In this way, a liquid sample or other liquid reagent initially applied to the sample zone 12 will be able to flow laterally from the sample receiving zone 12 into and through the label zone 14, into and through the capture zone 16, and finally into the absorbent zone 18 which acts as a wick or sink so that the entire sample or reagent volume may be flow through the zones 12, 14, and 16 in order to properly complete the assay.

In the preferred case of a nonbibulous assay device 10, both the sample receiving zone 12 and the label zone 14 will be composed of nonbibulous material which permit liquid flow in which all of the dissolved or dispersed component of the liquid are carried at substantially equal rates and with relatively unimpaired flow (in the absence of specific binding to a capture reagent as discussed hereinafter). Suitable nonbibulous materials include intrinsically nonbibulous materials, such as porous polyethylene, or intrinsically bibulous materials such as paper, nitrocellulose, polyesters, acrylonitriles, copolymers, rayon, and blends thereof, which

have been converted to exhibit nonbibulous flow characteristics by the application of blocking agents, such as detergents and proteins which inhibit the molecular forces which contribute to the bibulous nature of the material.

5 The sample receiving zone 12 serves to receive a small volume of the sample, typically in the range from 20 μ l to 200 μ l, and to initiate lateral flow to the label zone 14. The label zone 14 will typically include the target-specific labeling complex of the present invention impregnated or
10 otherwise immobilized therein. Usually, the labeling complex will be lyophilized as described above in connection with preparation of the test article. Flow of the sample or other reagent will then mobilize (solubilize) the labeling complexes and transport the complexes toward and into the capture
15 zone 16.

 It will be appreciated that there will be some interaction between the labeling complexes and an analyte within the sample which will mediate binding of the labeling complex within the capture zone 16. Such interaction can take
20 a variety of forms, typically being "sandwich" labeling (where the labeling complex includes anti-analyte which binds to the analyte and the capture zone includes immobilized anti-analyte which is then able to capture the labeling complex through the analyte) or "competition" labeling (where the labeling complex
25 comprises analyte or analyte analog and binding of the label to anti-analyte within the capture zone 16 is inversely proportional to the amount of analyte in the original sample). A variety of modifications to the basic sandwich and competitive assay formats are known in the art and can be
30 adapted for use with test devices identical or similar to test device 10 illustrated herein.

 The capture zone 16 may also be prepared from bibulous or nonbibulous materials, with nonbibulous materials being preferred. A particularly suitable material for the
35 capture zone is nitrocellulose which has been blocked to prevent bibulous interaction with the sample and other reagents.

In use, binding of the target-specific labeling complexes of the present invention within the capture zone 16 will provide a visual signal or indication which represents the assay results. Binding of a sufficient amount of the labeling complex within a defined location in the capture zone 16 will permit a user to visually read the result in the capture zone 16.

The following examples are offered by way of illustration, not by way of limitation.

10

EXPERIMENTAL

The following examples demonstrate preparation of a pre-dyed complexes for detection of human chorionic gonadotropin (hCG) in body fluids from an antibody-enzyme conjugate, appropriate enzyme substrate system, and the optional use of an enzyme inhibitor and/or a stabilizer.

15

EXAMPLE 1

1.1 Preparation of Reagents

1.1.1 Antibody Purification and Derivatization

Monoclonal anti-hCG ascites fluid was fractionated at 0-4°C by delipidation with sodium dextran sulfate and calcium chloride followed by ammonium sulfate treatment at 50% salt saturation and desalted on a G25 column into 10 mM Tris buffer (pH 8.0). The antibodies were further purified on a Q-Sepharose® FF resin using a salt gradient of 0 to 0.3 M sodium chloride in the same buffer. Fractionation was monitored at 280 nm, and the protein peak was collected and buffer-exchanged on a G25 column into 0.1 M sodium phosphate (pH 7.0). The resultant anti-hCG antibody was concentrated by ultrafiltration to 5 mg/ml.

30

Maleimide groups were introduced generally as described in *Enzyme Immunoassays*, Ishikawa et al., eds., Tgaku-Shoin, Tokyo - New York, 1981, into the anti-hCG antibody by adding maleimidobenzoyl-N-hydroxysuccinimide to a final concentration of 100 µg/ml, incubation of the reaction mixture for 45 minutes at 25°C, and buffer exchange on a G25 column into 0.1 M sodium phosphate (pH 6.0).

35

1.1.2 Preparation of Antibody-Enzyme Conjugates

To prepare antibody-enzyme conjugates, the following enzymes were used: glucose oxidase (GO), horseradish peroxidase (HRP), diaphorase I (DIA), and calf intestine alkaline phosphatase (ALP). To introduce SH groups, the enzymes were prepared at 5 mg/ml (GO, DIA or ALP) or 10 mg/ml (HRP) in 0.1 M sodium phosphate (pH 8.0) containing 0.5 mM 2-mercaptoethanol, incubated at 25°C for 45 min with 2-iminothiolane at a final concentration of 1.28 mg/ml, before being buffer-exchanged on a G25 column into 0.1 M sodium phosphate (pH 7.3).

The maleimide-containing monoclonal anti-hCG antibody (section 1.1 above) and the SH-derivatized enzymes were allowed to react for 2 hr. at 25°C followed by binding of the enzyme-antibody conjugate on a Sephacryl® S300 HR resin. A 0.45 µm filtered conjugate elution buffer composed of 0.05 M sodium phosphate buffer (pH 7.0) containing 0.1% (w/v) NaN₃, 0.03% (w/v), MgCl₂·6H₂O, 0.003% (w/v) ZnSO₄·7H₂O, and 0.005% (w/v) Tween® 20 was used throughout the fractionation. The fractionation was monitored at 280 nm, and the antibody-enzyme conjugate fraction pooled. Subsequently, the antibody-enzyme conjugates (except for anti-hCG/ALP conjugate) were buffer-exchanged on a G25 column into the following buffers supplemented with 50 µg gentamycin/ml: (i) HRP conjugate: 0.05 M Tris/HCl buffer (pH 7.5); (ii) GO conjugate: 0.1 M HEPES buffer (pH 8.0); and (iii) DIA conjugate: 0.1 M sodium phosphate (pH 8.0).

1.2 Preparation of Lateral Flow Assay Device for a "Wet" Two-Step Assay

Lateral flow nonbibulous assay test strip were constructed according to the methodology contained in co-pending application Serial No. 07/639,967. The test device (Fig. 1) includes three active zones and fourth absorbent zone which acts as a wick or sink to receive sample flow from the active zones. The active zones comprise a sample receiving zone 12, an intermediate zone 14, and a capture zone 16, as described below. The "wet" assay employs dye-label-complex prepared in a test tube, not illustrated, to which sample was

added. The resulting mixture was then applied to the test strip. Test strips were constructed as follows:

1.2.1 Preparation of Sample Receiving and Intermediate Zones

5 The sample receiving zone and intermediate zones were prepared from Sontara® 0-100 DuPont Orlon® spunlace fabric. The fabric was rendered nonbibulous by saturating with methylated bovine serum albumin (BSA). The conversion to nonbibulous material was achieved by treatment at $38 \mu\text{l}/\text{cm}^2$ with a 10 mg/ml solution of the BSA at room temperature at five minutes. The pad of Sontara® was then frozen at -70°C for at least an hour. The Sontara® fabric was then lyophilized overnight on a Virtis Freezemobile. The sample receiving pad and the intermediate zone pad were then cut into 10x4mm rectangles with the spunlace fibers being parallel to the longer side of each pad.

1.2.2. Preparation of Capture Zone Membrane

To prepare a nonbibulous capture zone, goat anti-hCG serum was fractionated at $0-4^\circ\text{C}$ by delipidation with sodium dextran sulfate and calcium chloride followed by ammonium sulfate treatment at 50% salt saturation and desalted on a G25 column into phosphate-buffered saline (pH 7.2; PBS). The anti-hCG antibodies were then further purified on an hCG-Sepharose® affinity resin using 0.1 M glycine buffer (pH 2.3) as the elution medium. Fractionation was monitored at 280 nm, the monospecific anti-hCG antibody peak collected and buffer-exchanged on a G25 column into PBS. Finally, the antibody was concentrated by ultrafiltration to $\geq 2 \text{ mg/ml}$.

Nitrocellulose having a pore size of $8 \mu\text{m}$ was affixed to an X-Y chart recorder, and hCG capture bands were formed as 2-cm spaced parallel lines by 2 mg/ml goat anti-hCG antibody using a plotter pen operated in the manual mode. These lines were reactive with hCG contained in a sample. After air drying for 10 min at room temperature, the nitrocellulose membrane was placed into a tray containing blocking buffer (10 mg/ml BSA in the above Tris buffer) for 15 min at room temperature. The membrane was removed, blotted, allowed to air dry, and subsequently stored in a desiccator at room temperature until assembly of the device.

1.2.3 Assembly of the Device

A 20 x 4 mm strip of the capture zone membrane was affixed centrally on an adhesive transparency strip. The transparency strip was a 70 x 4 mm strip of overhead projection
5 transparency film, made adhesive with double-sided adhesive tape. The intermediate zone was then affixed next to the capture zone pad with a 1 mm overlap. The sample receiving pad was then placed next to the intermediate pad with 1 mm overlap.

The device was then provided with an absorbent pad,
10 which was a 20 x 4 mm rectangle of cellulose paper which was affixed to the distal end of the capture zone membrane with a 1 mm overlap.

1.3 Preparation of Uninhibited, Pre-dyed Label Complex Containing Antibody-GO Conjugate for use in a Two-Step "Wet" Assay.

15

Uninhibited, pre-dyed label complex containing anti-hCG monoclonal antibody-GO conjugate was formed by a FAD-mediated electron transfer utilizing phenazine methosulfate as a chemical intermediate and a colorless hydrophylic tetrazolium
20 salt as an electron acceptor ("GO substrate"). This reaction results in a dyed enzyme-antibody conjugate suitable for detection of the analyte in the body fluid sample.

A 1.0 ml incubation reaction was carried out at 25°C by a sequential addition of the following components to 500 µl
25 of 0.1 M HEPES buffer (pH 8.0) containing 50 µg gentamycin/ml: (a) 100 µl of 8 mg MTT tetrazolium salt per ml of 50% (v/v) dimethylformamide (DMF) in deionized water; (b) 50 µl of 0.5 M D-(+)- glucose in the same HEPES buffer; (c) 50 µl of 0.5 mg PMS/ml of deionized water; and finally to initiate the complex
30 formation (d) 300 µl of anti-hCG antibody-GO conjugate at a final concentration of 1.7 to 50 µg/ml in the same HEPES buffer. At the time intervals specified in Table 1, 15 µl multiple aliquots were withdrawn, mixed with 25 µl samples of hCG standard (prepared in phosphate-buffer saline containing
35 0.5% (w/v) bovine serum albumin (BSA) at either 0 or 800 mIU/ml to give the final hCG concentration of either 0 or 500 mIU hCG/ml, respectively), and immediately applied to 4 mm wide lateral flow assay strips, prepared as described in section 1. The results based on the appearance of a visually discernible

positive signal in the capture zone after 5 minutes are reported in Table 1.

Table 1

5	GO-Ab, <u>μg/ml</u>	Time allowed (min.) <u>for Complex Formation</u>	hCG Concentration ¹ <u>in Standard</u>	
			<u>0 mIU/ml</u>	<u>500 mIU/ml</u>
10	1.7	10	-	-
		30	-	+/-
	5	10	-	-
		30	-	+
	50	10	-	-
		30	-	+
15	¹ + = Clearly discernible appearance of color in capture zone; +/- = Equivocal appearance of color in capture zone; and - = No discernable appearance of color in capture zone.			
20				

1.4 Preparation of Uninhibited, Pre-dyed Label Complex Containing Antibody-Alkaline Phosphatase (ALP) Conjugate for use in a Two-Step "Wet" Assay.

Uninhibited, pre-dyed label complex containing anti-hCG monoclonal antibody-ALP conjugate was formed by enzyme-catalyzed hydrolysis of a colorless organic phosphate ("ALP substrate") to a colored phenolic product ("ALP product"). A 550 μl incubation reaction was carried out at 25°C by a sequential addition of the following components: to 250 μl of 0.1 M AMPD (2-amino-2-methyl-1,3-propanediol) buffer (pH 10.5) containing 0.1% (w/v) NaN₃: (a) 200 μl of 5 mg BGP (Fast Bordeaux G diazonium salt) per ml of the same buffer coupled to 1-naphthol and (b) 45 μl of conjugate elution buffer, and finally to initiate the complex formation (c) 55 μl of anti-hCG-ALP conjugate in the conjugate elution buffer to the final concentration in the mixture of 2.75 μg or 5 μg/ml. After 1 min. incubation, 15 μl or 30 μl multiple aliquots were withdrawn, mixed with 25 μl or 10 μl aliquots of the hCG standard, respectively, to obtain a final concentration of hCG of 0 or 500 mIU/ml, and immediately applied to 4 mm wide lateral flow assay strips, prepared for a "wet" assay as described in section 1.2 above. Results based on the appearance of a visually discernible positive signal in the capture zone after 15 minutes are reported in Table 2.

Table 2

	ALP-Ab, <u>μg/ml,</u>	hCG Concentration in Standard ¹	
		<u>0 mIU/ml</u>	<u>500 mIU/ml</u>
5	1.02	-	-
	1.88	-	+
10	¹ + = Clearly discernible appearance of color in capture zone, and - = No discernible appearance of color in capture zone.		

Uninhibited, pre-dyed label complex containing antibody-ALP conjugate was also prepared by the indigo formation/tetazolium salt technique. A 600 μ l incubation reaction was carried out at 25°C by a sequential addition of the following components to 250 μ l of 0.1 M AMPD buffer (pH 10.5) containing 0.1% (w/v) NaN_3 : (a) 240 μ l of 2.16 mp IP (3-indoxyl phosphate disodium salt) per ml of 0.1 M AMP (2-amino-2-methyl-1-propanol) buffer (pH 10.15) containing 0.1% (w/v) NaN_3 ; (b) 10 μ l of 5 mg MTT/ml of 50% (v/v) DMF in deionized water, and finally to initiate the complex formation, (c) 100 μ l of anti-hCG antibody-ALP conjugate in conjugate elution buffer at a final concentration from 0.25 μ g/ml to 4.58 μ g/ml. After 1 min incubation, 15 μ l or 30 μ l multiple aliquots were withdrawn, mixed with 25 μ l or 10 μ l aliquots of the sample, respectively, to obtain a final concentration of hCG 0 or 500 mIU/ml, and immediately applied to 4 mm wide lateral flow assay strips, prepared for a "wet" assay as described in section 1.2 above. Results based on the appearance of a visually discernible positive signal in the capture zone after 15 minutes reported in Table 3.

Table 3

	ALP-Ab, <u>μg/ml,</u>	hCG Concentration in Standard ¹	
		<u>0 mIU/ml</u>	<u>500 mIU/ml</u>
35	0.313	-	-
40	0.625	-	+
45	¹ + = Clearly discernible appearance of color in capture zone, and - = No discernible appearance of color in capture zone.		

1.5 Preparation of Uninhibited, Pre-dyed Label Complex Containing Antibody-DIA for use in a Two-Step "Wet" Assay.

Uninhibited, pre-dyed label complex containing anti-hCG monoclonal antibody-Diaphorase (DIA) conjugate was formed by enzyme-catalyzed reduction involving NADH cofactor of a suitable organic hydrogen acceptor substrate to a product exhibiting disparate absorption spectrum in a visible region.

A 1677.5 μ l incubation was carried out at 25°C by a sequential addition of the following components to 445 μ l of 0.1 M sodium phosphate buffer (pH 8.0) containing 50 μ g gentamycin/ml (a) 67.5 μ l of 4.1 mg MTT in 15% (v/v) DMF in deionized water; (b) 67.5 μ l of 10 mg BSA (bovine serum albumin per ml of the same buffer); (c) 64.5 μ l of 10 mM NADH in the same buffer, and, finally to initiate the complex formation (d) 30 μ l of 50 μ g of anti-hCG monoclonal antibody-DIA conjugate in the same buffer. After 3 min incubation, 15 μ l multiple aliquots were withdrawn, mixed with 25 μ l samples of hCG standard to give the final hCG concentration of 0, 150 or 500 mIU hCG/ml, and immediately applied to 4 mm wide lateral flow assay strips, prepared for a "wet" assay as described in section 1.2 above. Results based on the appearance of a visually discernible positive signal in the capture zone after three minutes are presented in Table 4.

Table 4

	<u>hCG Concentration in the Standard</u>	<u>Result¹</u>
	0 mIU/ml	-
	150 mIU/ml	+
	500 mIU/ml	+

¹+ = Clearly discernible appearance of color in capture zone, and

- = No discernible appearance of color in capture zone.

1.6 Preparation of Uninhibited, Pre-dyed Label Complex Containing Antibody-HRP Conjugate for use in a Two-Step "Wet" Assay.

Uninhibited, pre-dyed label complex containing anti-hCG monoclonal antibody-HRP conjugate was formed by peroxide-mediated enzymatic conversion of colorless electron donor species to colored products. A 2016 μ l incubation reaction was

carried out at 25°C by a sequential addition of the following components to 360 µl of 3 mg of 4-chloro-1-naphthol per ml methanol: (a) 54 µl of 0.05 M Tris/HCl buffer (pH 7.5) containing 50 µg gentamycin/ml; (b) 1548 µl of 0.05 M Tris/HCl buffer (pH 7.5) containing 0.02% (v/v) H₂O₂, 0.1 mM EDTA (ethylenediaminetetracetic acid), and 50 µg gentamycin/ml; and finally to initiate the complex formation (c) 54 µl of 0.27 mg anti-hCG antibody/HRP conjugate in 0.05 M Tris/HCl buffer (pH 7.5) containing 50 µg gentamycin/ml. After 15 min incubation, the following reagents (chilled to 4°C) were added: (a) 126 µl of 0.05M Tris/HCl buffer (pH 7.5) containing 50 µg gentaycine/ml; (b) 126 µl of 0.05M Tris/HCl buffer (pH 7.5) containing 0.02% (v/v) H₂O₂, 0.1 mM EDTA and 50 µg gentamycin/ml; and (c) 252 µl 100 mg mBSA/ml in 0.05M Tris/HCl buffer pH 8.0. Multiple 15 µl aliquots were withdrawn, mixed with 25 µl samples of hCG standard to give final concentration of 0, 150 and 500 mIU hCG/ml; and immediately applied to 4 mm wide lateral flow assay strips, prepared for a "wet" assay as described in section 1.2 above. Results based on appearance of a visually discernible positive signal in the capture zone after three minutes are presented in Table 5.

Table 5

25	hCG Concentration in the Standard	Result ¹
	0 mIU/ml	-
	150 mIU/ml	+
	500 mIU/ml	+
30	1+ = Clearly discernible appearance of color in capture zone, and	
	- = No discernible appearance of color in capture zone.	

35

Uninhibited, pre-dyed complex containing the antibody-HRP conjugate was also prepared by reaction with electron-dense carbazole derivatives. HRP substrate was prepared by mixing of 50 µl of 0.40% (w/v) solution at 3-amino-9-ethylcarbazole, dissolved in dimethylformamide with 940 µl of 50 mM sodium acetate buffer (pH 5.0), followed by addition of 10 µl of 0.3% (v/w) hydrogen peroxide in the same buffer. A 5 µl aliquot of anti-hCG antibody/HRP conjugate (0.34 mg/ml); prepared as

40

described above in this section was added to 228 μ l of HRP substrate solution. Following 4 hr. incubation at 25°C, 10 μ l aliquots were withdrawn and immediately applied to 4 mm wide lateral flow assay strips assembled for "wet" assays as described in section 1.2 above. 20 μ l hCG samples prepared in pooled male urine were added onto the sample receiving strip pad to give a final standardized concentrations of 0, 100 or 400 mIU hCG/ml. Finally, 10 μ l portions of unspiked, apparently negative male urine pool were applied to the sample receiving zone to conclude the test. Results based on the appearance of a visually discernible positive signal in the capture zone after 8 minutes are reported in Table 6.

Table 6

15	<u>hCG Concentration in the Standard</u>	<u>Result¹</u>
	0 mIU/ml	-
	100 mIU/ml	+
20	400 mIU/ml	+
	¹⁺ = Clearly discernible appearance of color in capture zone, and	
25	- = No discernible appearance of color in capture zone.	

EXAMPLE 2

2.1 Preparation of Inhibited Pre-Dyed Label Complex Containing
 30 Antibody-HRP Conjugate for use in a Two-Step "Wet" Assay

Inhibited pre-dyed label complexes for detection of hCG in body fluids were constructed to demonstrate that the complexes need not possess enzymatic activity for detection of hCG in the samples once pre-dyed anti-hCG complex was formed.

35 Inhibited pre-dyed complexes are those where the activity of the enzyme in the initial antibody-enzyme conjugate has been inhibited following incubation of the antibody-enzyme conjugate with a suitable enzyme substrate to form the pre-dyed label complexes.

40 Inhibited, pre-dyed label complex containing anti-hCG antibody/HRP conjugate were prepared as follows. A 2016 μ l incubation reaction was carried out at 25°C by a sequential addition of 360 μ l of 3 mg of 4-chloro-1-naphthol per ml methanol of the following: (a) 54 μ l of 0.05 M Tris/HCl buffer

(pH 7.5) containing 50 μ g gentamycin/ml; (b) 1548 μ l of 0.05 M Tris/HCl buffer (pH 7.5) containing 0.02% (v/v) H_2O_2 , 0.1 mM EDTA (ethylenediaminetetracetic acid) and 50 μ g gentamycin/ml; and finally to initiate the complex formation (c) 54 μ l of

5 0.27 mg anti-hCG antibody/HRP conjugate in 0.05 M Tris HCl buffer (pH 7.5) containing 50 μ g gentamycin/ml. After 15 min incubation, the following reagents (chilled to 4°C) were added: (a) 125 μ l of 2 M NaN_3 in 0.05 M Tris/HCl buffer (pH 7.5) containing 50 μ g gentamycin/ml; (b) 126 μ l of 10% (w/v) PVP in

10 0.05 M Tris/HCl buffer (pH 7.5) containing 0.02% (v/v) H_2O_2 , 0.1 mM EDTA and 50 μ g gentamycin/ml; and 252 μ l 100 mg is BSA/ml in 0.05 M Tris/HCl buffer (pH 8.0). Subsequently, 15 μ l multiple aliquots were withdrawn, mixed with 25 μ l samples of hCG standard to give the final concentration of 0, 150 and 500

15 mIU hCG/ml; and immediately applied to 4 mm wide lateral flow assay strips, prepared for a "wet" assay as described above. Results based on the appearance of a visually discernible positive signal in the capture zone after 3 and 60 minutes are reported in Table 7.

20

Table 7

	<u>hCG Concentration</u> <u>in standard</u>	<u>Result</u> <u>at 3 min.¹</u>	<u>Result</u> <u>at 60 min.¹</u>
25	0 mIU/ml	-	-
	100 mIU/ml	+	+
	500 mIU/ml	+	+
30	¹ + = Clearly discernible appearance of color in capture zone, and - = No discernible appearance of color in capture zone.		

35 EXAMPLE 3

3.1 Preparation of Lateral Flow Assay Device for a "One-Step"
Assay for hCG Utilizing Uninhibited Pre-Dyed Label Complex

The test devices for a "one-step" assay were prepared

40 as described above for a "wet" two-step assay by replacing the intermediate zone of the latter with a pre-dyed label pad prepared as follows. Pre-dyed label complex containing antibody-HRP conjugate was prepared in a 2016 μ l incubation reaction at 25°C by a sequential addition to 360 μ l of 3 mg of

4-chloro-1-naphthol per ml methanol of the following: (a) 54 μ l of 0.05 M Tris/HCl buffer (pH 7.5) containing 50 μ g gentamycin/ml; (b) 1548 μ l of 0.05 M Tris/HCl buffer (pH 7.5) containing 0.02% (v/v) H_2O_2 , 0.1 mM EDTA and 50 μ g gentamycin/ml; and finally to initiate the complex formation; (c) 54 μ l of 0.27 mg anti-hCG antibody/HRP conjugate in 0.05 M Tris/HCl buffer (pH 7.5) containing 50 μ g gentamycin/ml. After 15 minutes incubation, the following reagents (chilled to 4°C) were added: (a) 125 μ l of 0.05M Tris/HCl buffer (pH 7.5) containing 50 μ g gentamycin/ml; (b) 126 μ l of 0.05M Tris/HCl buffer (pH 7.5) containing 0.02% (v/v) H_2O_2 , 0.1 mM EDTA and 50 μ g gentamycin/ml; and (c) 252 μ l of 100 mg mBSA/ml in 0.05 M Tris/HCl buffer (pH 8.0).

The mixture was poured onto Sontara spunlace fabric at 38 μ l/cm². The matrix was kept at room temperature for 20 minutes and abruptly frozen at -70°C, along with the lyophilization flask for at least an hour. The resulting composition was lyophilized overnight on Virtis Freezemobile, and the intermediate pre-dyed labeling pads were cut into 10x4 mm rectangles with the spunlace fibers parallel to the longer side of the pad. The pads were then assembled as intermediate zones into a "one-step" device in a manner analogous to that described above for the "wet" two-step assay strips.

Assays were performed by the addition of 40 μ l of 0, 150 or 500 mIU hCG/ml to the strip. Results based on the appearance of a visually discernible positive signal in the capture zone after 3 and 60 minutes are reported in Table 8.

Table 8

30	<u>hCG Concentration in Standard</u>	<u>Result at 3 min.¹</u>	<u>Result at 60 min.¹</u>
	0 mIU/ml	-	-
	100 mIU/ml	+	+
	500 mIU/ml	+	+
35	1+ = - =	Clearly discernible appearance of color in capture zone, and No discernible appearance of color in capture zone.	

Although the foregoing invention has been described in some detail by way of illustration and example, for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of
5 the appended claims.

WHAT IS CLAIMED IS:

1. A target-specific labeling complex comprising:
an enzyme bound to a specific binding substance; and
5 dye molecules complexed to the conjugate in an amount
sufficient to impart a visually discernable color to the
complex while leaving an active site of the specific binding
substance available for binding to a target molecule, wherein
the dye molecules were formed by reaction in aqueous phase of a
10 substrate with the enzyme bound to the specific binding
substance.
2. A labelling complex as in claim 1, wherein the
specific binding substance is an antibody.
- 15 3. A labelling complex as in claim 2, wherein the
enzyme is covalently attached to the antibody.
4. A labelling complex as in claim 1, wherein the
20 enzyme is selected from the group consisting of oxidases,
peroxidases, phosphatases, diaphorases, galactosidases, and
oxidoreductases.
5. A labelling complex as in claim 1, wherein the
25 enzyme is inactivated.
6. A labelling complex as in claim 1, wherein the
complex includes from 10 to 10^7 dye molecules.
- 30 7. A labelling complex as in claim 1, wherein the
complex is lyophilized.
8. A test article comprising:
a support matrix; and
35 a target-specific labeling complex impregnated within
the support matrix, wherein the complex comprises:
(i) a conjugate of an enzyme and a specific binding
substance; and

(ii) dye molecules complexed to the conjugate in an amount sufficient to impart a visually discernable color to the complex while leaving an active site of the specific binding substance available for binding to a target molecule.

5

9. A test article as in claim 8, wherein the target-specific labeling complex is lyophilized within the support matrix.

10

10. A test article as in claim 8, wherein the support matrix is a solid matrix which conducts nonbibulous flow of a liquid sample.

15

11. A test article as in claim 8, wherein the support matrix is a solid matrix which conducts bibulous flow of a liquid sample.

20

12. A test article as in claim 8, wherein the support matrix is a fabric composed of a polyester, an acrylonitrile copolymer, rayon, glass fiber, cellulose, and blends thereof.

25

13. A test article as in claim 12, wherein the fabric has been treated with a blocking agent to render the fabric nonbibulous.

14. A test article as in claim 8, wherein the specific binding substance is an antibody.

30

15. A test article as in claim 14, wherein the enzyme is covalently attached to the antibody.

35

16. A test article as in claim 8, wherein the enzyme is selected from the group consisting of oxidases, peroxidases, phosphatases, diaphorases, galactosidases, lytic enzymes, and oxidoreductases.

17. A test article as in claim 8, wherein the enzyme is inactivated.

18. A test article as in claim 8, wherein the complex includes from 10 to 10^7 dye molecules.

19. A test article as in claim 8, wherein the complex
5 is lyophilized.

20. A method for preparing target-specific labeling complexes, said method comprising.

providing a conjugate of an enzyme and a specific
10 binding substance, wherein the enzyme is capable of converting a substrate to a colored dye product and the specific binding substance is capable of binding to a target molecule; and
reacting the conjugate with the substrate in an
aqueous medium under conditions which result in the
15 complexation of the colored dye product with the conjugate.

21. A method as in claim 20, wherein the reaction conditions are selected to promote turnover of the substrate by the enzyme.

20

22. A method as in claim 20 wherein reaction between the conjugate and the substrate is stopped after the complex has obtained a desired color intensity.

25

23. A method as in claim 20, wherein the reaction is stopped by substrate exhaustion.

24. A method as in claim 20, wherein the reaction is stopped by altering the reaction conditions.

30

25. A method as in claim 20, wherein the reaction is stopped by inactivating the enzyme.

26. A method as in claim 20, wherein the specific
35 binding substance is an antibody.

27. A method as in claim 26, wherein the enzyme is covalently attached to the antibody.

28. A method as in claim 20, wherein the enzyme is selected from the group consisting of oxidases, peroxidases, phosphatases, diaphorases, galactosidases, lytic enzymes, and oxidoreductases.

5

29. In a device for detecting the presence of an analyte in a liquid sample, which device includes:

(a) a sample receiving zone comprising a solid support matrix which conducts lateral flow of liquid sample, in
10 contact with

(b) a labeling zone comprising a solid support matrix which conducts lateral flow of liquid sample and at least one assay label which specifically binds to or competes with the analyte, said labeling zone being in contact with

15 (c) a capture zone comprising a solid support matrix which conducts lateral flow and in at least a portion thereof at least one capture reagent capable of binding said assay label or analyte, said capture zone being contiguous with

(d) an absorbent zone,
20 the improvement which comprises an assay label comprising:

a conjugate of an enzyme and a specific binding substance; and

25 dye molecules complexed to the conjugate in an amount sufficient to impart a visually discernable color to the complex while leaving an active site of the specific binding substance available for binding to a target molecule.

30. A device as in claim 29, wherein the specific
30 binding substance is an antibody.

31. A device as in claim 29, wherein the enzyme is covalently attached to the antibody.

35 32. A device as in claim 29, wherein the enzyme is selected from the group consisting of oxidases, peroxidases, phosphatases, diaphorases, galactosidases, lytic enzymes, and oxidoreductases.

33. A device as in claim 29, wherein the enzyme is inactivated.

34. A device as in claim 29, wherein the complex
5 includes from 10 to 10^7 dye molecules.

35. A device as in claim 29, wherein the complex is lyophilized.

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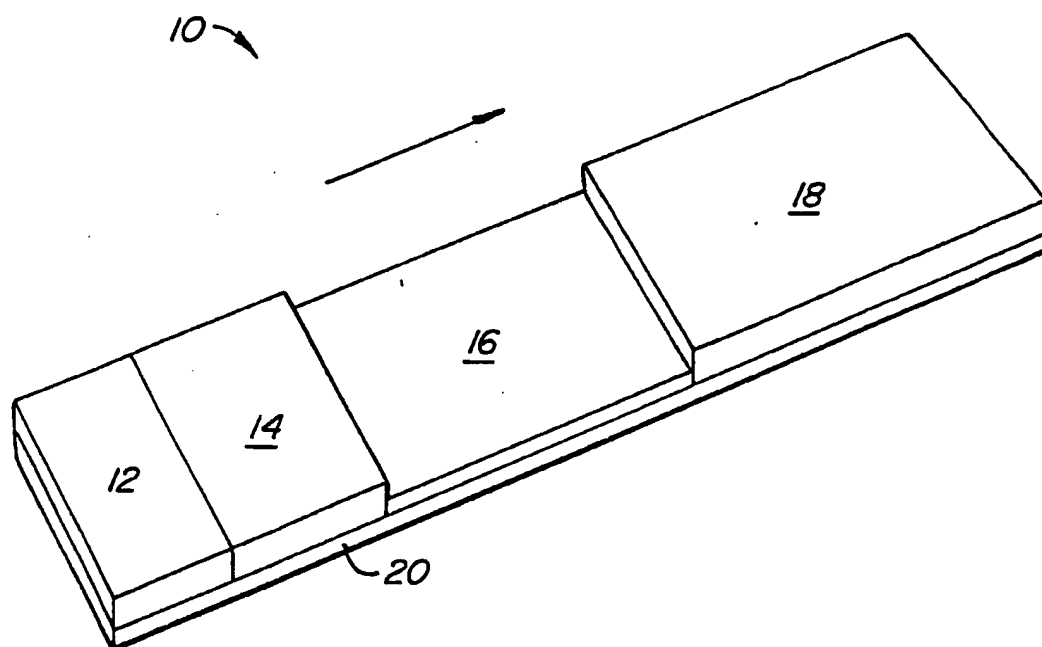


FIG. 1.

INTERNATIONAL SEARCH REPORT

L. National application No.
PCT/US93/06276

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) : IPC(5): GOIN 33/544, 33/542, 33/536

US CL : Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : Please See Extra Sheet.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
<u>X</u> Y	US, A, 4,786,589 (Rounds) 22 November 1988, col. 1, lines 47-59; col. 3, lines 55-56; col. 4, line 47 - col. 5, line 6; col. 5, lines 17-26; col. 7, lines 43-49; Examples III and V.	<u>1-28</u> 29-35
X,P	US, A, 5,137,804 (Greene et al) 11 August 1992, col. 12, line 22 - col. 14, line 17.	1-28
Y	US, A, 4,943,522 (Eisinger et al) 24 July 1990, abstract; col. 10, line 50 - col. 14, line 28.	29-35
Y	US, A, 4,861,711 (Friesen et al) 29 August 1989, Fig. 2; col. 2, line 37 - col. 7, line 13; col. 8, line 8.	29-35

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means		
"P" document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

24 AUGUST 1993

Date of mailing of the international search report

02 SEP 1993

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Authorized officer

CAROL A. SPIEGEL

Facsimile No. NOT APPLICABLE

Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US93/06276

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	GB, A, 2,204,398 (May et al) 09 November 1988, abstract; page 5, lines 10-24; page 8, lines 20-21 and 30-32.	29-35
Y	ANALYTICAL BIOCHEMISTRY, volume 169, issued 1988, H. Yasue et al, "Enhancement of the Sensitivity for <u>in Situ</u> Detection of Alkaline Phosphatase Using a Homopolymer of 2-Acrylamide 2-Methylpropanesulfonate", pages 410-414, especially page 411.	1-35

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/06276

A. CLASSIFICATION OF SUBJECT MATTER:

US CL :

422/56, 57; 435/7.9, 7.92, 7.93, 7.94, 7.95, 188; 436/518, 524, 527, 528, 529, 530, 531

B. FIELDS SEARCHED

Minimum documentation searched

Classification System: U.S.

422/56, 57, 60; 435/7.5, 7.6, 7.9, 7.91, 7.92, 7.93, 7.94, 7.95, 14, 18, 19, 21, 25, 28, 188, 805, 969, 970; 436/164, 169, 170, 501, 518, 524, 527, 528, 529, 530, 531, 810, 814; 427/2

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

DIALOG, APS, JPO

search terms: dye, formazan, indigo, indamine, label, marker, tag, immunoassay, enzyme, oxidase, peroxidase, phosphatase, diaphorase, galactosidase, oxidoreductase

